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TITLE: ANTIGEN AND GENOME DETECTION OF ARENAVIRUS, BUNYAVIRUS,
          AND FILOVIRUS INFECTIONS

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DISTRIBUTION STATEMENT: Approved for public release;
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93-13826

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93 6 18 036

REPORT DOCUMENTATION PAGE	Form Approved OMB No. 0704-0188

Antigen and Genome Detection of Arenavirus, Bunyavirus, and Filovirus Infections

5. FUNDING NUMBERS
Contract No.
DAMD17-88-C-8149

63002A
3M263002D807.AH.031
WUDA315324

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The specific aims of this project were to develop detection systems for recognizing and quanitating antigens and genomes of hemorrhagic fever viruses, and to use these detection systems to investigate the pathogenesis of these viruses in animal models. These were to be accomplished using existing antibodies and nucleic acid probes supplied by the MRDC as well as virus infected tissues or cell cultures.

Pichinde virus, an arenavirus non-pathogenic to humans, is a New World arenavirus. Previous reports indicate similarities between Pichinde infection in guinea pigs and Lassa fever infections in guinea pigs and monkeys. We examined Pichinde-infected Strain 13 guinea pigs to gain some insight into the pathogenesis of these viruses

Gene probes; Diagnosis; RAI; BD; Arenaviruses;
Bunyaviruses; Filoviruses

Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

FINAL REPORT: CONTRACT DAMD17-88-C-8149

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The antigen detection protocol, originally developed for identification of Pichinde-infected cells in formalin-fixed paraffin embedded (FFPE) specimens, was modified for the detection of Lassa fever viral antigens and is now used for detection of Lassa antigens at Fort Detrick.

With this methodology, the pathogenesis of arenaviral infections was explored. The enclosed manuscript, accepted by The American Journal of Tropical Medicine and Hygiene, describes our findings.

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1) DETECTION SYSTEMS

A. Antigen detection

Fixation

Seven fixatives were evaluated for their ability to adequately preserve Pichinde viral antigens while maintaining acceptable cellular morphology. Vero cells preparations infected with Pichinde virus strain 4763-1 were fixed in 4% paraformaldehyde, 10% Neutral buffered formalin (NBF), IEM (2.0% paraformaldehyde, 0.1% glutaraldehyde, sucrose, sodium phosphate), Zinc formalin, Omnifix (polyethylene glycol, acetic acid, ethanol), MBC (4.0% paraformaldehyde, 0.5% glutaraldehyde, calcium chloride, glucose, sodium cacodylate) and B-5 (mercuric chloride, sodium acetate, formilin) fixatives. Slides were stained by hematoxylin and eosin (H&E), and by immunoperoxidase using anti-Pichinde antibodies. No fixative proved to be superior to 10% NBF for the preservation of both viral antigens and cellular detail. Thus, the methodology developed for arenavirus antigen detection in FFPE blocks was based upon formalin fixation of infected tissues. Since formalin is the most common fixative in the laboratory worldwide, the immunocytochemical techniques described in this report are applicable to formalin-fixed archival tissues as well, with some

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modification. We found that fixation time has a direct effect on the ability to detect arenaviral antigens.

Proteolytic treatment is required to breakdown formalin induced crosslinking of the viral antigens. Our experiments defined the optimal digestion for Pichinde-infected guinea pig tissues, formalin-fixed for 4-5 hours, to be 50 ug/mL Proteinase K (Sigma Chemical Co.) for 30 minutes at 37°C.

We found extended fixation of infected tissues necessitates either increasing the length of the digestion step or increasing the concentration of the protease. Tissues from monkeys, infected with the Josiah strain of Lassa fever virus and fixed in formalin for 30 days, required a fourfold increase in protease concentration, 200 ug/mL Proteinase K, to successfully demonstrate Lassa viral antigen. Alternatively, the protease concentration may be increased to 500 ug/mL and the digestion time reduced to 3 minutes. The use of high concentrations of protease and/or extended digestion times requires tissues to be mounted on treated slides (ie.silanated, TES (3-aminopropyltriethoxysilane);Sigma, Superfrost Plus;Fisher Scientific) which promote adherence and minimize tissue loss.

Optimization of Immunocytochemical Staining

Pichinde Virus: The optimal procedure for staining Pichinde viral antigens in formalin-fixed paraffin embedded tissue is described in the enclosed manuscript.

For immunodetection of Pichinde viral antigens, we modified the standard indirect immunoperoxidase method by (1) adding a proteolytic digestion step, (2) extending the primary antibody incubation step to 16 hr. (overnight, 4°C), and (3) enhancing the 3,3'-diaminobenzidine tetrahydrochloride (DAB)-peroxidase endproduct with nickel chloride. These three modifications produced intense specific staining of virus infected cells without nonspecific background staining, and a sensitivity approaching that seen by immunofluorescent staining of frozen tissues.

In contrast, experiments conducted using biotinylated secondary antibodies and alkaline phosphatase-labelled streptavidin produced excessive background. The streptavidin bound nonspecifically to endogenous stores of biotin in the liver, a major arenavirus target. Moreover, endogenous alkaline phosphatase in sections of kidney and gut, resistant to treatment with levamisole, produced spurious staining in both infected and uninfected tissues.

The primary antibody, a hamster polyclonal anti-Pichinde antibody, was obtained from an outside source, McMaster University. Checkerboard titration of the primary and peroxidase

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conjugated secondary antibodies (goat anti-hamster IgG,

Kirkegaard & Perry Laboratories; #42206) determined the optimal

dilutions to be 1:200 and 1:50, respectively. Only two primary

anti-Pichinde antibodies were provided by USAMRIID, monoclonal P-

903 and anti-Pichinde guinea pig plasma 4763-1. Neither antibody

was amenable for use in FFPE tissue. The epitope recognized by

the P-903 antibody was denatured by formalin fixation. It was,

however, successful for detecting viral antigen in acetone-fixed

frozen tissues by both immunofluorescent and immunoperoxidase

techniques. The 4763-1 guinea pig plasma contained excessive

auto-antibodies resulting in high non-specific background

staining when applied to the experimental guinea pig tissues

(same species as antibody host).

Lassa Fever Virus: We applied the same indirect

immunoperoxidase method to archival FFPE sections from monkeys

infected with the Josiah strain of Lassa fever virus. Anti-Lassa

antibodies supplied by USAMRIID included pooled plasma #172

(Josiah, monkey), pooled plasma #2 (Josiah, guinea pig), pooled

plasma #7 (Macenta, guinea pig) and 6 monoclonal antibodies (L52-

121-22-BA02, L52-135-17A, L52-158-3A, L52-2159-15, L52-134-23-A,

L52-2074-7A, Josiah) specific for Lassa N and G proteins.

The Lassa infected tissues had been fixed in 10% NBF for

thirty days. We found it necessary to increase the Proteinase K

concentration to 200 ug/mL to "unmask" the viral antigens.

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Using pooled plasma #172 (Josiah, monkey) resulted in specific staining with excessive background, probably from contaminating auto-antibodies (same species source, tissue and antibody). Pooled plasma # 7 (Macenta, guinea pig) did not react with the Josiah strain infected tissues. Pooled plasma #2 (Josiah, guinea pig) detected viral antigens in spleen, liver, and lung but background staining was unacceptable. Absorption with Vero cells was insufficient in significantly reducing the background.

Specific staining for Lassa virus antigens was achieved by combining equal amounts of the six monoclonal antibodies (Moabs) into a cocktail and incubating the sections overnight (4°C, approx. 200uL/slide). The Moab cocktail was diluted 1:200 in PBS, pH 7.4. We used a high affinity peroxidase-conjugated goat anti-mouse IgG secondary antibody (#170-6516, Bio-Rad Laboratories) diluted 1:200 in PBS, pH 7.4. We were unable to compare the sensitivity for staining FFPE versus frozen tissues as no Lassa-infected frozen tissue was available. The antigen staining was highly specific for the Josiah strain of Lassa virus and there was no crossreactivity with the Macenta strain nor with Junin virus. Minimal crossreactivity was seen in epithelial cells of the gut, salivary gland and kidney tubules of infected and uninfected animals. This non-specific staining was perinuclear, possibly in the Golgi apparatus, and distinguishable from specific cytoplasmic staining of viral antigens. Specific

findings of antigen distribution in various tissues were described in our 14th Quarterly Report. The details of the Lassa protocol are described in the enclosed method (Method for Lassa on Paraffin Sections: Protease Digestion) adopted by the Pathology Division of USAMRIID and currently employed routinely by their Immunohistochemistry Laboratory to detect Lassa fever viral antigens.

We also evaluated the Antigen Retrieval System (ARS) distributed by Biogenex, Inc. for use in detecting Lassa viral antigens. This system employs microwaving slide mounted tissue sections in the presence of toxic Lead(II) Thiocyanate, as an alternative to proteolysis, for the unmasking of protein antigens crosslinked by formalin fixation. After adjusting the microwave time required to expose Lassa antigens we produced the same staining pattern in infected liver sections as seen by protease treatment. However, results were not always reproducible and varied depending on the type of tissue. For example, when liver, spleen, lung, and bowel sections were processed together, lung and bowel sections were poorly stained, with increased background staining, while liver and spleen sections were well stained with only minimal background. This was not seen in batched tissues subjected to Proteinase K treatment. Thus, the inability to batch specimens combined with the inconsistency of the results led us to abandon this technique.

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Double Immunofluorescence (manuscript in progress)

To further classify specific cell types sensitive to Pichinde virus infection we performed double immunofluor ₌ent experiments on frozen tissues using anti-Pichinde antibodies and antibodies specific for endothelial cells (Factor VIII-related antigen), guinea pig IgG, guinea pig macrophages (MR-1) and vascular smooth muscle (alpha-actin). Secondary antibodies were conjugated to either fluorescein or rhodamine.

Polyclonal antibody to human Factor VII-related antigen (DAKO 082, diluted 1:200) reacted with guinea pig endothelial cells. The MR-1 antibody (Serotec MCA-518, diluted 1:500) reacted with infected and uninfected guinea pig macrophages in the gut, spleen and lung. Guinea pig IgG antibody (DAKO F233, diluted 1:200) co-localized with Pichinde viral antigens in renal glomeruli suggesting trapping of antigen-antibody complexes within the basement membrane.

B. Genome Detection

MRDC did not provide any cloned viral genetic material. Four cDNA clones representing overlapping segments of the S region of the Pichinde genome were obtained from Dr. David Auperin of the Centers for Disease Control. These clones were the original clones used by Auperin et. al. to sequence the Pichinde viral genome. One of the clones, designated A3-89, encodes the open reading frame (ORF) for the viral nucleocapsid protein. A second

clone, 1-243, encodes the ORF for the viral glycoprotein precursor. The remaining two clones encode regions of extensive secondary structure (hairpin loop) in the viral genome and were not used. Since viral nucleocapsid protein appears to be the most abundant protein in arenaviral infections, we chose the A3-89 clone for initial probe synthesis.

Two concurrent approaches to the in situ demonstration of the Pichinde genome were undertaken. In both approaches all solutions were treated with Diethyl pyrocarbonate(DEPC; Sigma) to inhibit RNAase activity. Glassware was baked at 260°C for at least 2 hours.

The first approach involved non-isotopic detection using DNA probes. Synthesis of DNA probes was accomplished by labelling the entire A3-89 plasmid as well as have PCR-generated smaller fragments within the nucleocapsid open reading frame. The DNA was labelled with biotin-11-dUTP by nick translation, by adding biotin-11-dUTP directly to the PCR mixture, or with digoxigenin-UTP by random priming.

We used Pichinde-infected Vero cells from the same harvest that yielded optimal results by antigen detection to determine the proper hybridization conditions. Vero cells infected with Pichinde virus as well as uninfected control cells were harvested and spotted on TES coated slides. At first, cells were harvested at day 7 of infection but later studies used cells at day 10-11

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after demonstration of increased signal with immunofluorescent antibody staining after 10 days of infection.

In addition, frozen and paraffin embedded tissues, fixed in either 10% NBF or 4% paraformaldehyde, were also tested.

1.Northern blot

Estimation of the genome copy number in the Pichinde-infected Vero cells was determined by Northern blot analysis. Briefly, total cellular RNA was extracted from one 75 cm^2 flask of infected Vero cells as well as one control flask of uninfected Vero cells (approx. 3 x 10^6 cells per flask). Samples of the RNA were run on a denaturing formaldehyde gel and blotted onto a nylon filter. The filter was probed with the entire A3-89 plasmid labelled with ^{32}P by random priming. Results from the Northern blot show a strong positive viral band in all of the infected lanes without any evidence of hybridization to the control RNA. The amount of Pichinde viral RNA was estimated to be 100 pg per lane which corresponded to 1/100 of the total RNA. This translates into an approximate genome copy number of 100-300 for day 10 infected Vero cells.

2.DNA Probes

Biotinylated Probes

Experiments tested the efficacy of various pretreatment regimens, the effect of probe concentration, and the effect of varying the

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detection system used for all of probe preparations. In cases
using biotinylated Pichinde probes, a biotinylated positive
control probe consisting of total human DNA (BRL Life
Technologies, Inc.) was used along with the virus probe.
Positive results were obtained with a nick-translated
biotinylated A3-89 probe using a high (>10 mg/ml) probe
concentration, minimal pretreatment of slides, and the optimal
colorimetric detection system (BRL kit).   The BRL in situ
hybridization kits, along with the majority of the published
protocols, use a Streptavidin-alkaline phosphatase conjugate in
the detection step. This system was not ideal for Vero cells due
to the endogenous alkaline phosphatase activity of the cell line.
Although positive results were obtained with this method, the
results are not easily reproducible and difficult to quantitate
because the color development must be very precisely controlled
to prevent obscuring of the signal by the endogenous activity.
Additionally, DNA probe concentrations in the ranges published
for DNA-DNA nonisotopic hybridizations were found to be
insufficient for generation of signal. The signal was also
obscured with predigestion of cells and extensive pretreatments.

     To circumvent the problem of endogenous alkaline phosphatase
in Vero cells, streptavidin conjugated to beta-galactosidase was
tried. The experiments were repeated using a streptavidin-beta
galactosidase conjugate, an enzyme not present in eukaryotic

cells. Results were uniformly negative and no signal could be obtained with the positive control probe.

Horseradish peroxidase-labelled probes

Plasmid A3-89 DNA was grown at Digene, Inc. and labelled by conjugation with horseradish peroxidase (HRP). Provided with the labelled DNA were reaction conditions and solutions used by Digene for DNA-DNA hybridizations. The reaction conditions were tested using plasmid PL-2, containing the entire BPV-1 (bovine papilloma virus) genome as probe. The target was a frozen section of bovine fibropapilloma fixed 5 minutes in acetone. Briefly, the steps involved included blocking of endogenous peroxidase with H_2O_2, washes in denaturing buffer, application of the probe in Digene hybridization cocktail, heat denaturation, hybridization for 45 minutes at RT, washing, and detection with DAB. This protocol yielded positive results in all BPV sections.

For hybridization to the Pichinde RNA target using the A3-89 probe, the above reaction conditions were varied to include Proteinase K digestion in varying amounts (0.5 ug/mL to 100 ug/mL, 3 to 10 minutes), an incubation in 2X SSC at 70°C for 30 minutes, and omission of the 100°C heat denaturation step. Probe concentrations were varied from 5.0 ug/mL to 50 ug/mL. No signal was obtained on acetone-fixed Pichinde-infected Vero cells or on FFPE tissue sections from Pichinde-infected animals.

Further experiments utilized HRP protocols developed at ENZO Diagnostics designed originally for the detection of HIV RNA in fixed tissue. This procedure involved Proteinase K digestion, postfixation in 4% paraformaldehyde, prehybridization in 50% formamide/2X SSC, and hybridization in a buffer of 2X SSC, 50% formamide, 15% dextran sulfate, carrier DNA, and 10 ug/mL probe. Hybridization was performed for 90 minutes at 37°C. Following washes, detection was done using DAB. No positive signal could be generated using this protocol.

Digoxigenin-labelled probes

Experiments were also conducted using digoxigenin-labelled RNA probes synthesized by following the protocol developed by Boehringer Mannheim. We ligated a 351 bp fragment of the A3-89 clone into the multiple cloning site of Promega's SP-70 plasmid which is also flanked the T7 and SP6 phage promoters. Various manipulations designed to optimize probe stability, target stability, probe concentration, hybridization buffer content, facilitate probe entry (proteolysis). hybridization temperature, stringency of posthybridization washes and chromogenic development were unsuccessful in achieving a consistent positive signal.

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3.RNA Probes

Concurrent with the nonisotopic approach was been an attempt to identify the viral genome using single stranded ^{35}S-labelled riboprobes. Such techniques have been most successful in the **in situ** demonstration of RNA both in our previous experience with other systems and in published reports.

In separate experiments large scale preps of pBR322 containing either the A3-89 or 1-243 clones were grown for the generation of Pichinde NP-specific and GP-specific Riboprobes. Following the large prep, the bacteria were lysed and the plasmid DNA was recovered and purified using the QIAGEN resin columns or, in some cases, by cesium chloride gradient centrifugation. The 408 bp XmnI fragment of the 1-243 clone (GPC) was excised by restriction digest, purified and subcloned into the SP70 (Promega) transcription vector. In other experiments, a 358 bp PvuII fragment from A3-89 was subcloned into the PvuII site of SP70. This vector also allows a DNA insert to be cloned into a site flanked by phage promoter sites (T7 and SP6). The orientation of each insert was determined by restriction fragment analysis and the presence of each insert was confirmed on polyacrylamide DNA sequencing gels. Both constructs were used to transform HB-101 competent cells. Following transformation the cells were grown on LB/ampicillin plates (100ug/mL ampicillin) and colonies were selected and screened for the presence of the constructs (SP70 confers ampicillin resistance) using Stratagene

miniprep columns and agarose gel electrophoresis. Briefly, 10 colonies of each (A3-89, 1-243) were selected and grown up overnight in LB broth. The bacteria were centrifuged, resuspended in lysis buffer containing lysozyme and RNase and recentrifuged. The pellet of bacterial debris was removed. The supernatant containing the desired DNA was added to the Stratagene column and pushed through at a flow rate of 1 drop/second. The column eluate was precipitated with an equal volume of isopropanol and pelleted. The pellet was washed in 70% ETOH, dried and resuspended in TE buffer. Each of the samples was digested with PVU II under the appropriate conditions and then run out on a 0.8% agarose gel to check for the presence the inserts. One colony containing one of each of the respective inserts was isolated and grown up again in large scale preps. Others were frozen down for future use if needed.

The large prep (SP70/1-243, SP70/A3-89) DNA was recovered and purified, again, via the QIAGEN column method and checked by restriction digest with PVU II and gel electrophoresis to confirm the presence of the insert. The DNA concentration of each sample was determined by mass spectrophotometry. Before we transcribed the ^{35}S labelled probes we again sequenced the inserts compare our sequences with those published by Auperin et al., confirming that the clone we received was correct and secondly, to determine which of the DNA strands is virus-sense and which is virus complementary-sense.

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^{35}S labelled Riboprobes were transcribed using Promega's in vitro Gemini system, following the manufacturer's instructions. 50 uCi of ^{35}S UTP (Amersham, Inc.) was added for incorporation into each of the probes. Isotopic labelling of each probe was verified by scintillation count and only probes with counts greater or equal to 5 x 10^5 were used for in situ experiments.

Following the protocol of Oncor, Inc. we performed in situ hybridization of frozen sections of Pichinde-infected tissues fixed for 5 minutes in 4% paraformaldehyde. Pretreatments included 10 minutes in triethanolamine/acetic anhydride, 30 minutes in Tris-glycine, 2X SSC washing, dehydration through graded alcohols and air drying. Sections were hybridized at 52°C for 3 hours using 1 part 10^5 CPM/uL A3-89 probe and 9 parts Oncor RNA hybridization mix. Post hybridization treatments included 2X SSC washes, with and without 50% formamide, at 52°C, and RNase incubation to remove unhybridized probe. Slides were dehydrated, air dried and exposed to NTB-2 emulsion (Eastman Kodak CO.) for 10 days. Sections were developed in D-19 developer, fixed in Kodak fixer, washed in distilled water, air dried and counterstained with hematoxylin.

This protocol yielded positive signal primarily in macrophages of spleen, liver and lung. Riboprobes were tried on archival FFPE tissue blocks without success. The fixation of cells has been shown by many authors to be a critical parameter in RNA retention. Reports find paraformaldehyde to be the

fixative of choice for RNA targets. Short fixation times also seem to be important in RNA detection.

Adequate preservation of target RNA and protection from RNase contamination is essential. Isotopic RNA probes were the most sensitive in this study, however proper disposal of radioactive waste is becoming increasingly more difficult. The recent introduction of more sensitive non-isotopic kits, particularly Boehringer Mannheim's latest digoxigenin based 'Genius' kits will inevitably become the method of choice.

2) PATHOGENESIS OF PICHINDE VIRUS

We requested and received approval to conduct sequential sacrifice studies of guinea pigs infected with Pichinde virus strain 4763-1. Strain 13 guinea pigs, infected with Pichinde virus have been portrayed, by Jahrling et al., as an animal model for Lassa fever virus infection in humans. To follow the progression of the infection, infected animals were sacrificed approximately every other day. We examined sixteen different tissues from each animal by the immunohistochemical method described above. We identified the sequence by which different organs become infected, and the specific cells types within each organ containing Pichinde viral antigen. The enclosed manuscript, Pathogenesis of Pichinde Infection in Strain 13 Guinea Pigs: An Immunocytochemical, Virological and Clinical Chemistry Study, Connolly et al. reports the results of those

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studies. It has been accepted for publication in The American Journal of Tropical Medicine & Hygiene.

A second manuscript is in progress which will detail the results of our double labelling immunofluorescent experiments.

A third manuscript, Pichinde Virus-Induced Respiratory Failure Due to Constrictive Obstruction of the Small Airways: Structure and Function, Schaeffer RC, Bitrick MS, Connolly B, Jenson AB and Gong FC, has been submitted for publication. This arose out of collaborative efforts with the Department of Physiology, Univ. of Arizona Health Sciences Center, Tucson, AZ.

A fourth manuscript is also underway in conjunction with Dr. Richard J. Montali, Department of Pathology, National Zoological Park, Smithsonian Institution, Washington D.C.. Here, we applied our immunoperoxidase assay to FFPE tissues of endangered golden lion tamarins and marmosets infected with a newly described strain of LCMV (J of Virology 1991. 65,3995-4000). This represents the first time these antigens have been detected in formalin-fixed tissues.

METHOD FOR LASSA ON PARAFFIN SECTIONS : PROTEASE DIGESTION

Step 1. Deparaffinization

Place two experimental slides plus two Lassa (Josiah) antigen positive Rhesus monkey slides from accession #870388 or #870386; and two normal Rhesus monkey slides accession #911221 into a 25 slide rack. Place the rack into Xyless® (3 changes for 3 mins. each), 100% ETOH (3 changes for 3 mins. each), 95% ETOH (3 changes for 3 mins. each), distilled water (2 changes approximately 2 mins. each). Agitate slides in each reagent.
During this time you should make up:

A. Protease solution

183 ml 0.1M Na2HPO4 (Sodium Phosphate, Dibasic)
17 ml 0.1M NaH2PO4   (Sodium Phosphate, Monobasic)
0.1000 g of solid protease. (from freezer)

Combine 183 ml 0.1M Na2HPO4 (Sodium Phosphate, Dibasic) with 17 ml 0.1M NaH2PO4 (Sodium Phosphate, Monobasic) to give 200 ml of a pH 7.8 phosphate buffer. Add 0.1000 g of protease and place in a water bath at 37°C until needed.

B. 1% Milk PBS

100 ml PBS
1.0 g Carnation® Powdered Milk
Mix well (shake or use magnetic stirrer) and filter using Whatman® #3 filter paper.

C. Preparation of 3% methanol block:

20.0 ml 30% hydrogen peroxide
180.0 ml methanol
Make up one 200 ml block for each rack of 25 slides.

Step 2. Methanol block

Place slides in methanol block for 15 mins.

While slides are in the methanol block, prepare the proteinase-K solution as follows:

Preparation of proteinase-K solution:

Prepare 20 µl aliquots of proteinase-K stock solution: Add 1 ml of distilled water to 20 mg (0.02 grams) of proteinase-K. Vortex the solution to mix. Divide into 20µl aliquots and store in freezer at   20°C. Thaw and use individual aliquots as needed (do not refreeze).

Into a 20 ml vial combine one 20 µl aliquot (from above) of the proteinase-K solution and 3980 µl of PBS. Vortex to mix. Place in 37°C waterbath for 10-15 mins. to pre-warm the solution. (be sure cap is on tight and use partially water filled small beaker to hold the vial upright in the waterbath).

Place small humidity chamber (containing enough PBS to cover bottom of the chamber) in the oven at 37°C for 10-15 mins. to pre-warm the chamber.

STEP 3. Distilled water rinse
Rinse slides in distilled water (3 changes for 3 min. each).

STEP 4. PBS rinse
Rinse slides once in PBS for 3 min.

Step 5. Proteinase-K digestion:
Place slides horizontally in humidity chamber and place 100µl (use pipette) of the pre-warmed proteinase-K solution on each slide and coverslip (be sure no air bubbles are under coverslip). Place cover on humidity chamber and incubate in oven for 30 mins. at 37°C. (alternative method is to mix 20 mg of proteinase-K with 200 ml of PBS and place slides in this solution for 30 min. at 37°C).

During this incubation time prepare serum block as follows:

Preparation of 10% normal goat serum block:
500 µl goat serum + 4.5 ml PBS 1% milk. Vortex to mix.

At end of incubation period, individually dip slides in a beaker of PBS and pull slide straight out. This will remove the coverslips.

Step 6. PBS rinse
Rinse slides in PBS (3 changes for 5 mins. each).

Step 7. Normal goat serum block
Shake if the slides and carefully wipe around the tissue. Place 10% normal goat serum block on each slide and incubate at room temperature for 20 mins. During this incubation period prepare the 1:200 dilution of the primary antibody monoclonal cocktail as follows:

Preparation of primary antibody dilution of mouse anti Lassa monoclonal cocktail:
The monoclonal cocktail (antibody log #531) is made of equal volumes of 6 monoclonal mouse antibodies identified below:
    L52-121-22-BA02   Antibody log # 279

L52-134-23-A	Antibody log # 487
L52-135-17-A	Antibody log # 488
L52-158-3-A	Antibody log # 490
L52-2159-15	Antibody log # 499
L52-2074-7A	Antibody log # 495

Make a 1:200 dilution of this antibody cocktail using PBS (no milk). Assume use of 200 µl of primary antibody per slide. Use this   information to calculate the to: volume needed. Vortex the   solution to mix. Example: 5 µl of 1° Ab cocktail plus 9⁣: µl of PBS,   or 10 µl of 1°Ab coctail plus 1990 µl of PBS.

Step 8. Primary antibody (mouse anti Lassa monoclonal cocktail)

Shake the serum block off each experimental slide. Wipe around each tissue. Place 200 µl of the 1:200 dilution of the primary antibody on each experimental slide. (leave serum block on one positive control slide). Incubate overnight in the refrigerator at 4°C in a humidity chamber. Be sure chamber is level in the refrigerator.

Step 9. PBS rinse

Take humidity chamber out of refrigerator. Take lid off of chamber and let warm up at room temperature for 15 min.

Rinse slides in PBS (3 changes for 4 min each).

During the PBS rinses, prepare the secondary Ab as follows:

Preparation of secondary Ab:

Make a 1:200 dilution of peroxidase conjugated goat anti mouse IgG (antibody log #532) using PBS (Example: 10 µl of goat anti mouse   peroxidase conjugated IgG and 1990 µl PBS). Vortex to mix.

Step 10. Secondary antibody:

Shake off the slides and carefully wipe around the tissue. Using a 1:200 dilution of the secondary antibody prepared above, place 200 µl on each slide and incubate in humidity chamber for 30 min.

Step 11. PBS rinse

Rinse slides in PBS (3 changes for 4 min. each).

Step 12. DAB NiCl$_2$ chromagen

Make up chromagen solution during the last PBS rinse.
a. Make a H$_2$O$_2$ solution using 5 µl of 30% hydrogen peroxide and 500 µl of distilled water.

b. Using a 20 ml vial mix 10 mg of DAB and 20 ml of 1X DAB buffer solution containing $NiCl_2$ (working dilution) . Shake and vortex to mix. Filter through a 0.2 micrometer Nalgene syringe filter (using a 25-30 cc syringe) into a clean beaker.
c. Add one drop of 3% H_2O_2 (made in step 10a) to 20 ml of DAB-DAB buffer with NiCl (made in step 10b) Vortex and use immediately.

Shake off slides, and wipe carefully around the tissue. Cover tissue on each slide solution made in step 10c and incubate in humidity chamber at room temperature fo 5 to 10 min (examine slides under microscope at 10X after 5 min and thereafter to determine optimum time). Once optimum chromagen decoration has occurred, decant reagent from each slide into beaker (dispose of solution into DAB waste container).

Step 13. Tap water rinse
Immediately rinse slides in running tap water for 4 mins. (don't use distilled water there will be yellowing of slides).

Step 14. Nuclear fast red staining
Place slides directly from tap water r...se into nuclear fast red stain (use straight from bottle) for 5 mins.

Step 15. Tap water rinse
Decant nuclear fast red stain back into bottle (for reuse) and rinse slides in running tap water for 1-2 mins.

Step 16. Dehydration
Dehydrate slides through alcohol to Xyless:
95% ETOH (2 changes, 20 dips each)
100% ETOH (2 changes, 20 dips each)
Xyless (2 changes, 20 dips each)

Step 17. Coverslip
Wipe slides to remove excess Xyless and coverslip using permount.

*Developed by Brett Connolly and A.B. Jenson, Georgetown University

Short Title: Pichinde Virus Infection in Strain 13 Guinea Pigs

Pathogenesis of Pichinde Infection in Strain 13 Guinea Pigs: An Immunocytochemical, Virological and Clinical Chemical Study.

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ABSTRACT

Pichinde virus has been adapted to produce infection of Strain 13 guinea pigs. Viral replication and presence of viral antigen in frozen tissues stained by immunofluorescence has been previously described. Further investigation into the of pathogenesis disease has been hampered by the lack of a light microscopic method for correlating histologic lesions and the presence of Pichinde viral antigens. For this purpose, we developed a sensitive immunocytochemical technique for staining Pichinde viral antigens in formalin-fixed paraffin embedded tissue. Enhancement of the immunocytochemical staining with nickel chloride markedly improved detection of viral antigens. We examined frozen and formalin-fixed tissues from Strain 13 guinea pigs for viral antigens by light microscopy and immunocytochemistry at various intervals after infection with Pichinde virus. Progressive involvement of different tissues correlated with organ injury measured by serum biochemical abnormalities. Pichinde viral antigen was first detected in splenic macrophages 5 days after infection and their subsequent destruction facilitated persistent viremia. The inability to clear virus led to multiple organ infection and vascular involvement. Ensuing infections involved particularly liver, spleen, adrenals, lung, and intestines. Gastroenteritis developed with extensive involvement of the muscularis mucosa throughout the gastrointestinal tract. Water and food intake decreased rapidly after day 8, leading to marked weight loss. Fatty change of the liver suggested metabolic derangement that was further exacerbated terminally by adrenal infection and pulmonary impairment.

INTRODUCTION

The Arenaviridae are lipid-enveloped negative-stranded RNA viruses that cause chronic, nonlethal infections in natural rodent hosts.[1] Five of the 13 recognized arenaviruses are human pathogens. One of these, lymphocytic choriomeningitis virus (LCMV), transmitted by asymptomatically infected house mice, causes disease in humans who contact the virus vis rodent excreta. In humans LCMV usually causes only a mild febrile aseptic meningitis, but severe or even lethal encephalitis with involvement of other organs may occur.[1,2] Other well known but less characterized human pathogens (Lassa, Machupo, and Junin) produce a severe, often fatal, hemorrhagic fever syndrome.[3-7] Maximal containment is required for experimental work with these virulent agents. Recently, a new arenavirus, provisionally designated Guanarito virus, was recently identified as a cause of hemorrhagic fever in Venezuala.[8]

Pichinde virus (an arenavirus nonpathogenic for man) has been adapted to cause lethal infection in Strain 13 guinea pigs.[9] This experimental model has similarities to Lassa Fever virus infection in both humans and guinea pigs.[1,7,9,10]

To better understand the pathogenesis of arenavirus infections, we used immunocytochemical staining of viral antigens to identify particular cell types infected by Pichinde virus in Strain 13 guinea pigs. We also correlated histopathologic changes in infected cells with serum biochemical abnormalities that indicated specific organ damage.

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MATERIALS AND METHODS

Cell Culture and Infection

Vero cells were propagated in monolayer cultures at 37°C under 5% CO/2\ with Eagle's MEM containing 10% fetal bovine serum and supplemented with glutamine, penicillin, and streptomycin. Monolayers of cells were infected with Pichinde virus strain 4763-1 at approximately 1-5 infectious units per cell. The inoculum was the supernatant from previously infected cells lysed by freezing and titrated for Pichinde virus infectivity by immunofluorescence.

Eleven days after infection, cell monolayers from 75-cm\2/ culture flasks were harvested by trypsinization, washed three times in phosphate buffered saline (PBS), pelleted at 2000 x g, and resuspended in 1 mL of sterile PBS. Infected or uninfected cells were spotted directly (5 µL) or applied by cytocentrifugation (Shandon Inc., Pittsburgh, PA; 1:1000 dilution of cell suspension) onto acid-cleaned glass slides coated with 3-aminopropyltriethoxysilane (TES, Sigma Chemical Co., St. Louis, MO) to enhance adherence. These preparations were air-dried for 1 hour. Slides were fixed in acetone for immunofluorescent staining or in one of the fixatives for immunoperoxidase staining. After fixation, slides were air-dried, placed in dust-free boxes, and stored at -70°C until stained.

Guinea Pigs

Female inbred Strain 13 guinea pigs weighing 350-550 gm, from Crest Caviary (Mariposa, CA), were acclimated for 1 week before inoculation. Animals were maintained in individual cages at an ambient air temperature od 22-25°C, and a 12-hour light dark cycle.

Twenty-six guinea pigs were inoculated intraperitoneally with 2 x 10\3/ PFU of adapted Pichinde virus in 0.2 mL of a suspension prepared after 12

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serial guinea pig spleen passages (Dr. Peter Jahrling, USAMRIID, Ft. Detrick, Frederick, MD); 10 uninfected animals served as controls. Infected animals were housed in a Horsfall Isolator Unit (Plas-Labs, Lansing, MI) and monitored twice daily for physical signs of illness. Uninfected animals were housed in a separate room.

Control and infected guinea pigs were weighed daily, and the weights averaged for each group. Both groups were provided with food and water for consumption |ad libitum|. Water consumption was determined by measuring the difference between a full water bottle (500 mL) and the amount remaining 24 hours later. The amount of dry food consumed was calculated by weighing the food needed to refill calibrated feeders each day.

Guinea Pig Tissues

One control and at least two infected animals were sacrificed on days 1, 3, 5, 7, 9, 10, 11, 13, 17, 18, and 20. Organs were removed aseptically within a laminar-flow safety cabinet. Liver, spleen, Pancreas, kidney, adrenal, heart, lung, brain, thymus, salivary gland, urinary bladder, stomach, and small and large intestines were processed for frozen sectioning and immunocytochemistry and, in some cases, reserved for virus titration. A portion of each organ was embedded in OCT (optimal cutting temperature) compound (Miles Laboratories, Inc., Elkart, IN), snap frozen on dry ice, and stored at $-70°C$ until 6-μm sections were cut with a cryostat. A portion of each brain, spleen, and liver was also snap frozen and stored at $-70°C$ until assayed for infectious virus. The remainder of each tissue was immediately fixed in 10% neutral buffered formalin (NBF) for 4 to 5 hours, processed in a Histomatic tissue processor (Fisher Scientific Co., Pittsburgh, PA), and embedded in paraffin for cutting 4-μm sections and staining with hematoxylin

and eosin (H&E) or by immunoperoxidase.

Viral Plaque Assay

Serum for assay of infectious virus was separated from blood obtained via cardiac puncture, by centrifugation at 1500 x g, and stored at -70°C until used. Spleen, liver, and brain samples were suspended (10% wt/vol) in Eagle's medium with nonessential amino acids, 5% fetal bovine serum, and antibiotics. After homogenation in tissue grinders (Tenbroek, SGA Scientific Inc., Bloomfield, NJ), the suspensions were clarified by centrifugation at 800 x g for 30 minutes and stored at -70°C until assayed.

Viral titers were quantitated by inoculating 50 µL of serial 10-fold dilutions onto duplicate 16-mm\2/ monolayers of Vero cells in 24-well plates (Costar, Inc.,Cambridge, MA). After 1 hour of adsorption, 0.6 mL of 0.5% agarose in MEM containing Earl's balanced salt solution with 8% heat-inactivated fetal bovine serum and 150 µg/mL gentamicin was added to each well. The plates were incubated for 4 days at 37°C in a 5% CO/2\ humidified incubator. On day 4, 0.6 mL of 0.1 mg/mL neutral red (Grand Island Biological Co., Grand Island, NY) was added to visualize plaques. Foci were counted on day 5, and viral titer was expressed as PFU/gm (tissues) or PFU/mL (serum).\9/

Immunofluorescence

Indirect immunofluorescent staining of acetone-fixed Vero cell preparations and frozen sections of guinea pig organs was performed by using as primary antibody either murine monoclonal antibody P903 (USAMRIID) specific for Pichinde virus nucleocapsid protein or hamster polyclonal antiserum recognizing Pichinde virus nucleocapsid protein and viral envelope glycoproteins (kindly provided by the late Dr. William Rawls, McMaster

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University). The secondary antibody was either goat anti-murine or anti-hamster, respectively, conjugated with either fluorescein or rhodamine. Slides were examined with an Olympus BH-2 fluorescence microscope equipped with a mercury light source and epi-illumination.

Checkerboard titration was used to determine dilutions of antibodies for optimal staining and minimal background fluorescence. Negative controls included omitting the primary antibody, substituting for the primary antibody with nonimmune serum of the same species or with an antibody specific for an unrelated antigen (e.g., papillomavirus), and staining uninfected Vero cells or guinea pig tissues with the correct combination of primary and secondary antibodies.

Fixation

Several fixatives were evaluated for preservation of both cellular morphology and viral antigenic determinants (Table 1). The fixatives tested included 4% paraformaldehyde, 10% NBF, IEM, zinc formalin, MBC, Omnifix, and B-5. Slide preparations of Pichinde infected Vero cells were treated with each fixative for to minutes and stained by indirect immunoperoxidase methods using the P903 Moab of the hamster polyclonal antibody. To yield detectable staining, all slides prepared with these fixatives required proteolytic treatment with proteinase K (Sigma Chemical Co.).

Immunocytochemistry

Formalin-fixed paraffin embedded (FFPE) tissues were cut into 4 μm sections and baked onto glass slides at 60°C for 30 minutes; paraffin was removed with xylene, and the sections were hydrated with dH$_2$O for indirect immunoperoxidase staining.\11-14/ Sections were immersed in 3% H$_2$O$_2$ for

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10 minutes and washed in water. Sections were rinsed in 0.05 mol/L PBS, pH 7.2, and digested at 37°C with 0.1 mL of prewarmed proteinase K (50 μg/mL; Sigma Chemical Co.).\11/ After three PBS washes, sections were covered with 10% nonimmune goat serum (DAKO Corp., Carpinteria CA) containing 1.0% bovine serum albumin (Sigma Chemical Co.) followed by incubation with the polyclonal anti-Pichinde virus antibody (diluted 1:200 in PBS) overnight at 4°C in a humidified chamber. Sections were then brought to room temperature, Washed in PBS, and covered with peroxidase-conjugated goat anti-hamster IgG (diluted 1:50 in PBS; Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 30 minutes. The peroxidase was developed with 0.5 mg/mL 3,3′diaminobenzidine tetrahydrochloride (DAB) substrate (Polysciences Inc, Warrington, PA) solubilized in buffer containing NiCl/2\ (Digene Diagnostics, Silver Spring, MD) for 5-10 minutes. Sections were washed in dH/2\O, counterstained with Nuclear Fast Red (Sigma Chemical Co.). dehydrated, cleared, and mounted with coverslips. Slides were examined with an Olympus BH-2 microscope equipped with an Olympus C-35AD-4 35 mm camera.

Serum Chemistry Analysis

Glucose, uric acid, blood urea nitrogen (BUN), creatinine, sodium, potassium, chloride, bicarbonate, calcium, phosphorus, total protein, albumin, cholesterol, triglycerides, total bilirubin, direct bilirubin, and alkaline phosphatase were measured in serum samples collected by cardiac puncture. These measurements were made according to manufacturer's instructions with a Hitachi 737 automated chemistry analyzer using reagents from Boehringer Mannheim (Indianapolis, IN). The commercial calibrators were supplemented with enzymatic sources from animal tissues, thus these assays should be valid for samples form humans or animals. Potassium, aspartate aminotransferase,

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alanine aminotransferase, and lactate dehydrogenase were also measured but excluded from analysis because of artifacts in these analytes introduced by hemolysis in many of the samples.

RESULTS

Clinical Observations

Intake of food and water by infected guinea pigs was dramatically reduced beginning on day 8. Food intake was reduced by approximately 85% and water intake by about 65% throughout the remainder of the experiment, resulting in a progressive decline in weight (Fig. 1).

Activity decreased with weight loss. Animals surviving through day 13 were lethargic and had rapid, shallow breathing; fur was ruffled and slobbering was common. The first death from infection occurred on day 12, and at least one animal died from infection on each of days 13 to 17. All infected animals either died from infection or were sacrificed at one of the indicated timepoints.

At necropsy, no guinea pig exhibited signs of overt hemorrhage. From day 10 on, livers of infected animals were enlarged and had a yellow-brown discoloration.

Viral Replication

Viremia was detected as early as day 3, although viral titers in blood were significantly less than in spleens and livers of the same animals (Fig. 2). Titers from all sources increased throughout the course of infection. The viral titer in brain homogenates probably derived from blood perfusing the brain as no viral antigen was detected in the brain parenchymal cells by immunofluorescence or immunocytochemistry.

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Immunofluorescent Staining

Acetone-fixed Vero cells infected with Pichinde virus and stained by immunofluorescence with either monoclonal or polyclonal anti-Pichinde virus antibody revealed a fine, granular cytoplasmic pattern of intense fluorescence (Fig. 3). Similar patterns were seen in frozen sections of acetone-fixed Pichinde virus-infected guinea pig tissues. Uninfected Vero cells and tissues consistently failed to stain by immunofluorescence using either antibody.

Optimization of Immunocytochemical Staining

While immunofluorescence permitted assessment of the distribution of Pichinde viral antigens and the relative quantities in different organs, we sought to identify, by light microscopy, the specific cell types involved.

Using immunoperoxidase, we detected Pichinde viral antigens in Vero cells preserved with each of the fixatives. Fixing with 10% NBF, paraformaldehyde, and IEM resulted in better antigenic staining than did zinc formalin, Omnifix, MBC, and B-5 (Table 1). We used 10% NBF for subsequent experiments.

Initial efforts of immunoperoxidase staining of FFPE tissues resulted in difficulty in determining the endpoint of the DAB-peroxidase reaction. Extending the development time increased background staining without substantially improving the signal. Heavy metals such as nickel, copper, or cobalt are known to increase sensitivity and color intensity of DAB staining, with nickel providing a high signal-to-noise ratio.\15,16/ Staining for Pichinde viral antigen in FFPE tissues was markedly enhanced by solubilizing the DAB in a buffer containing NiCl/2\. The concentration of NiCl/2\ was proprietary information with the manufacturer; however, published reports

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indicate a working concentration of 0.7 mg/mL.\17,18/ By this means, Pichinde infected cells showed black, granular cytoplasmic staining identical in distribution to that seen with immunofluorescent staining.

The P903 Moab failed to detect Pichinde antigen in FFPE tissues, probably because of alteration or denaturation of the single epitope during fixation and/or processing. Consequently, all studies on FFPE tissues were performed with the polyclonal antibody.

To assess sensitivity, we compared immunocytochemical staining of FFPE tissues with immunofluorescent staining of frozen tissues. The distribution of Pichinde antigen was the same in both tissues, but approximately 15% fewer cells were stained in the FFPE tissues. This difference is likely due to two factors: routine processing of the tissue may alter or destroy some of the epitopes recognized by the polyclonal antibody, and the proteolytic digestion required for optimal immunocytochemical staining may eliminate some epitopes while making most of the remaining epitopes accessible for antibody binding.

Distribution of Viral Antigen

Results of Pichinde antigen detection in FFPE tissues by immunoperoxidase are summarized in Table 2.

Liver. The first abnormality detected in the liver by light microscopy consisted of mild fatty change associated with small foci of hepatic degeneration and necrosis seen on day 9. By day 11, moderate fatty change occurred with focal degeneration and necrosis and, possibly, Kupffer cells, but without inflammation. Occasional hemosiderin-laden Kupffer cells were apparent by day 13, Thereafter, morphological changes in the liver did not increase in severity, and fatty change decreased over days 17-20.

Immunoperoxidase staining for Pichinde antigen revealed occasional small

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foci of positive Kupffer cells and rare foci of positive hepatocytes by day 5 (Fig. 4). The number of positive Kupffer cells increased during days 7-13, whereas the number of positive hepatocytes increased throughout the infection. Viral antigen was present in both vacuolated (fatty change) and nonvacuolated hepatocytes with no apparent correlation between fatty change and antigen presence. By day 13, positive antigen staining of portal triad stromal cells was readily apparent and well-demarcated by the limiting plate and vascular bundle. Rarely, biliary epithelial cells were also positive. Variability among infected animals for both number and distribution of immunoperoxidase-positive hepatocytes was common during the later stages. Guinea pigs with high viral titers in liver had extensive hepatocellular antigen staining at days 17-21 (Fig. 4).

Spleen. On days 3 and 5, H&E staining revealed several discrete foci of large, pale cells containing phagocytized cellular material at the periphery of some splenic follicles. At day 7, almost all follicles contained variable foci of necrosis, and necrosis of perifollicular dendritic macrophages was evident. By days 9-10, perifollicular necrosis was more pronounced and was accompanied by numerous neutrophils. Erythrophagocytosis by red pulp macrophages was now prominent.

At days 11-14, numerous hemosiderin-laden macrophages were noted throughout the red pulp in areas previously showing erythrophagocytosis. Stains for iron were markedly positive in these macrophages. There was also a significant decrease in perifollicular macrophages concurrent with increased red pulp necrosis. Fibrin usually surrounded the splenic follicles, sporadically extending into the red pulp.

After day 14, the red pulp appeared disorganized and depleted of erythrocytes, with hemosiderin-laden macrophages much more prevalent than seen

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previously. The splenic follicles had multiple necrotic foci, and approximately half the animals showed spleens with prominent vascular channels lined by hypertrophied endothelial cells, many of which were viral-antigen positive.

Immunocytochemical staining demonstrated Pichinde antigen at day 5 to be confined predominantly to perifollicular macrophages and within scattered foci of reticuloendothelial cells of the red pulp. No antigen was detected within the white pulp. The number of antigen-positive red pulp foci increased by day 7 as did the number of dendritic macrophages (Fig. 4). Additionally, approximately 5-10% of the white pulp lesions had demonstrable antigen.

Spleens on days 9-21 showed increasing numbers of antigen-positive red pulp cells, primarily macrophages and endothelial cells. After day 13, approximately 50% of the hemosiderin-laden macrophages were positive for viral antigens. Only 10-20% of the white pulp lesions stained positive. Isolated reticular cells within the white became focally positive after day 13.

Animals examined on days 19 and 21 had extensive infections in their spleen. Many antigen-positive endothelial cells were seen lining prominent vascular channels throughout the red pulp (Fig. 4). Importantly, at all stages of infection, lymphocytes within the splenic follicles were antigen-negative.

Kidney. No tubular or glomerular lesions were seen in the kidney. There was no evidence of inflammatory infiltrates. Viral antigen was detected first at day 11 in a few small cortical foci of peritubular interstitial cells. Several glomeruli contained 1-3 positive mesangial cells, and occasional positive foci were seen in the transitional epithelial cells covering the minor calyx.

No significant changes were seen until day 17, when the number of antigen-positive peritubular foci increased. However, the number of positive

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cells was estimated to be less than 5% of the total population. Most of the cells within these foci were endothelial cells. Interlobular blood vessels also contained Pichinde antigen discernable in endothelial cells, vessel-wall smooth muscle cells, and underlying connective tissue.

Glomerular involvement remained unimpressive until day 18, when approximately 50% of cortical glomeruli contained variable numbers of antigen-positive mesangial cells. On day 20, viral antigen was abundant and almost exclusively confined within mesangial cells of cortical glomeruli.

Adrenal gland. H&E stained sections of adrenals revealed small foci of degenerative cell changes in the |zona fasiculata| by day 7. Neutrophils were seen in some of these degenerative foci, which became more extensive, reaching a peak at day 18. Dilatation of the capillary bed began on day 13, but no association was apparent between these dilated vessels and the areas of necrosis or the antigen-positive cells. In animals examined after day 13, the overall architecture of the gland was remarkable for its disorganization and disruption of the regular pattern of radial columns and plates.

By immunoperoxidase staining, Pichinde antigen was detected at day 7 not only in the degenerative foci, but also in normal appearing spongiocytes (Fig. 5). Antigen-positive were predominantly with the |zona fasiculata| early and within the |zona reticularis| and |zona glomerulosa| by day 13. Although variability among animals was seen at later times, in some animals greater than 90% of cortical spongiocytes contained viral antigens (Fig. 5).

Lung. H&E sections of lungs on day 7 showed perivascular edema of some small pulmonary veins and mild congestion of the alveolar capillary beds. At day 11, congestion and edema were slightly increased, and a proteinaceous was in some alveolar sacs. An abnormal number of mononuclear cells, some appearing marginated, were seen within venules, arterioles, and capillaries.

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At 13 days, focal areas of consolidation had thickened alveolar septa with mononuclear cell infiltrates and edema, consistent with a focal interstitial pneumonia. Despite negative Brown & Brenn stains, a bacterial infection could not be ruled out. Small blood vessels contained increased numbers of these mononuclear cells that appeared to partially obstruct the lumens.

Immunoperoxidase staining of day 7 specimens revealed sporadic Pichinde antigen-positive alveolar macrophages within alveolar air spaces and septa. By day 11 viral antigen was detected in many alveolar macrophages, in cells provisionally identified as type II pneumocytes, and in mesothelial cells lining the pleural surface. Hilar lymph nodes now contained antigen-positive macrophages within the subcapsular and medullary sinuses.

After day 15, there was a marked increase in antigen-positive cells within interalveolar walls throughout the parenchyma. The intensity of the stain presented some difficulty to differentiate between infected endothelial and interstitial cells. Pleural mesothelial cell infection extended to include approximately 80% of these cells.

By day 17, in addition to the expanding parenchymal involvement, there were large foci of antigen-positive tracheal and bronchial (large and small) epithelial cells (Fig. 5). Endothelial cells of some small pulmonary arterioles, clearly delineated by the internal elastic lamina, were also focally positive.

Gastrointestinal tract. Infiltrates of histiocytes and foci of necrosis (predominantly within villus tips) were apparent throughout the gastrointestinal (GI) tract, particularly the ileum, by day 9, becoming prominent by day 11. Many of these foci exhibited extensive karyorrhexis. Epithelial cells covering the villi showed no morphologic abnormality.

Immunostaining of various portions of the GI tract, particularly the

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small intestine, revealed histiocytic cells positive for viral antigens intermixed with cellular debris in the |lamina propria| of intestinal villi as early as day 7. Moreover, by day 13, smooth muscle cells of the |muscularis mucosa| were strongly positive (Fig 6.). Mucosal and submucosal glandular epithelial cells were negative at all stages of infection although the supporting stromal cells were markedly positive for viral antigen after day 13. During the later stages of infection (days 17-20), antigen-positive cells remained in the villus tips. Histiocytes of the |lamina propria| continued to contain antigen as did the |muscularis mucosa| and the adjacent stromal cells. However, staining intensity and the number of positive cells appeared to diminish.

Heart. There were no lesions or inflammatory processes identified in sections of the heart. Immunocytochemical staining identified a few small discrete foci within ventricular endocardial cells on day 13. In the myocardium, few fibroblasts in the connective tissue were positive. At days 18-20, random foci within the connective tissue of the heart valves were positive. Endothelial cells of some coronary vessels and myocardial capillaries also contained viral antigen. Cardiac muscle fibers contained no viral antigen.

Pancreas. H&E sections of pancreas were unremarkable until day 11 when there was mild cytoplasmic vacuolization of the acinar exocrine cells. This progressed to involve nearly 60% of the acinar cells by day 13, and 80-85% by day 17. There was no evidence of inflammation or necrosis.

By immunoperoxidase, few fibroblasts within the inter- and intralobular connective tissue septa were positive at day 11. From days 13-20, these foci became more numerous. Some cells within isolated pancreatic islets became positive at day 13. The numbers of antigen-positive islets and positive cells

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within each islet increased at days 18 and 20, but only 5% of the total number of islets were involved.

Urinary bladder. The bladders of guinea pigs at days 17, 18, , and 20 were distended and filled with dark, tea-colored urine. H&E sections from all bladders were unremarkable.

Viral antigen, consisting mainly of several scattered foci within the |lamina propria|, was first detected at day 7 in one of two animals. A solitary focus involved the transitional epithelium. By day 9, positive cells were also detected in the interstitial connective tissue of the muscularis. At day 13, Pichinde antigen was apparent on the serosal surface and in foci of bladder epithelium.

Vascular involvement was evident in the smooth muscle walls of small arterioles by day 14 (Fig. 6). Antigen staining was more extensive throughout the |lamina propria| and epithelium of two animals at day 17. On days 18 and 20, viral antigen was confined to the serosal surface and mucosal arterioles.

Blood Vessels. Vascular involvement by Pichinde virus was seen in sections of lung, heart, kidney, urinary bladder, mesenteric vessels, and vessels associated with the GI tract. Typically, antigen did not appear until day 13 and then in moribund animals. Immunostaining demonstrated positive cells within the |tunica media| of small arterioles as well as positive endothelial cells in venules and arterioles. In some arterioles, both antigen-positive and antigen-negative endothelial cells appeared rounded and protruded into the luminal space.

Thymus. H&E sections of thymus showed no abnormality until day 13, when the cortex appeared much thinner than in control animals or infected animals from earlier timepoints. By days 17-20, the cortex appeared almost absent and thymic lymphocytes were predominantly large, with pale-staining nuclei and

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little cytoplasm.

No antigen was seen in the thymus until day 10, when there were several positive foci of large cells within the medulla. These foci were more numerous at days 11-14, typically surrounding Hassall's corpuscles. At days 17-20, antigen staining intensity increased as these foci expanded. In general, the positive staining cells had large nuclei and abundant cytoplasm. Mature thymic lymphocytes appeared negative.

Brain. The brains of infected guinea pigs were unremarkable except for mild perivascular edema what may have been an artifact of tissue processing. Pichinde antigen was detected late in the infection in circulating monocytes within cranial blood vessels, but not within brain tissue.

Salivary gland. All salivary glands appeared normal and were viral-antigen negative throughout the study.

Serum Chemistry Analyses

Pichinde virus-infected animals showed dramatic loss of sodium and chloride beginning about day 7 (Fig. 7), consistent with acute adrenal cortical insufficiency and failure to secrete adequate mineralocorticoids. Beyond day 15, sodium and chloride concentrations apparently returned to normal when the animals became severely dehydrated as evidenced by weight loss, decreased water intake and markedly elevated BUN with only minor increase in serum creatinine (i.e. pre-renal-azotemia). Normal values of sodium and chloride in the face of extensive dehydration suggests depletion of whole body electrolytes as a late manifestation of adrenal cortical failure. The drop in bicarbonate after day 13 (Fig. 7) was consistent with metabolic acidosis induce by renal retention of phosphates, which almost doubled terminally in infected animals coincident with dehydration.

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Serum proteins decreased abruptly around day 10, mainly because of albumin loss (Fig. 8). Subsequently, albumin remained low whereas total rose to baseline values, perhaps because of compensatory release of acute-phase reactant proteins. The major drop in albumin was likely due to impaired synthesis by the liver resulting from decreased dietary intake and malabsorption in the extensively infected intestines. It was less likely due to proteinuria as the animals became nearly anuric as water intake dropped.

The transient four-fold elevation of triglycerides around day 10 (Fig. 8) coincided with both the fall in protein and a modest transient elevation of unconjugated bilirubin from baseline values of 0.2 - 0.3 mg/dL to a peak on days 9 and 10 of 1.1 - 1.2 mg/dL. Cholesterol showed a transient two-fold increase with triglycerides and terminal elevation with alkaline phosphatase (Fig. 8) suggesting biliary obstruction. These multiple biochemical findings suggest hepatic insult such as from starvation and caloric deprivation.

DISCUSSION

Previous investigations of Pichinde virus infection in guinea pigs have demonstrated tissue distributions of viral antigen by immunofluorescent staining and titers of infectious virus recovered from various tissues.\9/ Other work has been unsuccessful at revealing sufficient histologic change to account for death.\19/ This study extended those earlier findings by identifying the cellular targets of Pichinde virus in the Strain 13 guinea pig. The increased sensitivity of detecting viral antigen in formalin-fixed tissues by a NiCl/2\-enhanced immunoperoxidase method made possible a comparison of Pichinde viral antigen accumulation with histologic changes identified by H&E staining and standard light microscopy.

In general, minimal to moderate histopathologic lesions correlated with

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the presence of viral antigens. In adrenal, bladder, and liver viral antigen was detected in cells that appeared histologically normal, whereas presence of viral antigen corresponded to substantial histologic changes in the spleen, GI tract and lung. The sequence of infections involved splenic macrophages and Kupffer cells as initial targets (days 5-7), followed by foci of hepatocytes, adrenal spongiocytes of the |zona fasiculata|, alveolar macrophages and pneumocytes, histiocytes and muscularis mucosa in the GI tract, pancreatic islet cells, and urinary bladder epithelium (days 7-17). During late-stage infection (days 17-20), viral antigen was present on renal glomeruli, endothelial cells of various organs, and respiratory epithelium. Jahrling, et al.\9/ demonstrated similar patterns of Pichinde virus antigen by immunofluorescence, but without identification of infected cell types.

Particularly important in the sequence of infection was involvement of circulating and fixed tissue histiocytes as primary targets of Pichinde virus. This feature has been observed in other animal models.\20,21/ We identified Pichinde viral antigen as early as day 5 in perifollicular macrophages of the spleen. Viral antigens were subsequently identified in 30 to 50 percent of splenic macrophages, where extensive erthrythrophagocytosis resulted in hemosiderin accumulation. Because this phenomenon did not occur in control animals, it may reflect an inability of infected splenic macrophages to clear iron as happens in ascorbic conditions of the guinea pig.\22/ Most macrophages at other sites, such as lymph nodes, lungs and kidney (mesangial cells) were also infected at late stages.

This extensive infection of macrophage populations, particularly in the spleen, may have blunted the immune response thereby facilitating progression of the disease. In similar infections with LCMV, infected macrophages that process and present MHC-1 molecules with viral epitopes may be quickly

destroyed by cytotoxic T-cells.\23/   In this study, infection (days 5 to 7) and marked decrease (days 9 to 13) of perifollicular macrophages in spleens of Pichinde-infected guinea pigs may have allowed persistent viremia to develop and the infection to spread to other organs and cell types.

As with other reports of arenavirus infection,\17,19/ our study suggests that Pichinde virus causes lethal organ damage and blood chemistry abnormalities by interfering with the physiologic functions of various tissues without inflicting demonstrable injury on the majority of involved cells. The most obvious histopathologic abnormality, detected by light microscopy, was fatty change in the liver that appeared on day 9. Fatty change may be related more to the nutritional status of infected guinea pigs than to direct viral infection of hepatocytes. Infected guinea pigs stopped eating and drinking at roughly the same time that fatty change appeared. More than other animals, guinea pigs (even uninfected) depend on a daily intake of food and water for physiologic homeostasis. In addition, most vacuolated hepatocytes did not stain positively for Pichinde viral antigen, suggesting an indirect effect of infection on hepatocytes. Only in the late stages of infection, when fatty change of the liver was less prominent, were the majority of hepatocytes infected.

Although we did not directly measure virus shedding in urine, feces, or bronchial secretions, our results suggest that horizontal transmission of Pichinde virus in guinea pigs may be accomplished by shedding from extensively infected bronchial epithelium and focally infected transitional cells of the urinary bladder. Intestinal epithelial cells did not appear to harbor virus that would lead to fecal transmission.

This experimental model of Pichinde virus infection has demonstrated primary involvement and probable destruction of antigen-presenting

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macrophages, thereby providing the basis for persistent viremia and multiple organ infection. A unique finding from this study was the extensive involvement of adrenal cortex with concurrent loss of electrolyte control suggesting severe impairment of mineralocorticoid secretion. Widespread viral infection and replication in liver, adrenal, intestine and other organs induced little or no inflammatory response as in other arenaviral infections. Organ function was affected either directly through infection (malabsorption by intestine, mineralocorticoid regulation by adrenals) or as a secondary metabolic response (starvation, fatty change in liver, dehydration). These findings may be generally applicable to arenavirus infections and may warrant investigation of similar organ involvement in human infections not presently recognized due to lack of specific histopathologic changes.

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TABLE 1. Effect of Proteinase K Digestion on Immunoperoxidase Staining of Pichinde-Infected Vero Cells.

FIXATIVE\a/	Proteinase K digestion\b/ (min.)							
	0	2-4	4-6	6-8	8-10	10-12	12-14	>14
4% Paraformaldehyde	−	+	++	+++	++	−	−	−
10% NBF	−	+	++	+++	++	+/−	−	−
IEM	−	+/−	++	+++	++	+	−	−
Zinc formalin	−	+/−	+	++	+	−	−	−
Omni	−	+\−	+	+	−	−	−	−
MBC	−	−	+	+	+	−	−	−
B-5\c/	−	−	−	−	−	−	−	−

(−) no staining, (+) weak staining, (++) moderate staining, (+++) strong staining
\a/Vero cells were fixed in each fixative for 10 minutes at room temperature.
\b/Proteinase K digestion; 5 ug/mL 0.05 mol/L Tris buffer, pH 7.2 at 37\o/C.
\c/Minimal staining emerged after 25 minutes of Proteinase K treatment.

TABLE 2. Immunocytochemical Detection of Pichinde Viral Antigen in Formalin-Fixed, Paraffin-Embedded Tissue.

<u>Pichinde Infected</u>

<u>Days after Inoculation</u>

	1	3	5	7	9	10	11	13	14	17	18	20
Brain\a\	−	−	−	−	−	−	+/−	+/−	+/−	+/−	+/−	+/−
Thymus	−	−	−	−	−	+/−	+	++	++	++	+++	+++
Salivary	−	−	−	−	−	−	−	−	−	−	−	−
Heart	−	−	−	−	−	−	−	+/−	+	+	+	+
Lung	−	−	+	+	+	++	++	++	+	+++	+++	+++
Liver	−	−	+	++	++	++	++	+++	+++	++	+++	++
Spleen	−	−	+	++	++	++	++	+++	+++	+++	+++	++
Pancreas	−	−	−	−	−	−	+	+	++	++	++	++
Urin. Blad.	−	−	−	+	+	+	+	+	+	++	++	++
Kidney	−	−	−	−	−	−	+	++	++	++	++	++
Adrenal	−	−	−	+	++	++	+++	+++	+++	++++	++++	++++
Stomach	−	−	−	+	+	+	+	+	++	++	++	++
Duodenum	−	−	−	+	+	++	++	+++	+++	+++	++	++
Jejunum	−	−	−	+	+	++	++	+++	+++	+++	++	++
Ileum	−	−	−	+	+	++	++	+++	+++	+++	++	++
Colon	−	−	−	−	+	++	++	+++	+++	+++	++	++

\a\Antigen detected within circulating mononuclear cells.

Figure Legends

Figure 1. (A) Food consumption, (B) water consumption, and (C) weight loss, expressed as percent of initial weight, of Pichinde infected and uninfected Strain 13 guinea pigs. Points are arithmetic means, standard error < .05.

Figure 2. Pichinde viral titers in spleen, liver, brain (PFU/gm) and serum (PFU/mL) of Strain 13 guinea pigs inoculated i.p. with 2×10^3 PFU Pichinde virus. Points are geometric means, standard error < .05.
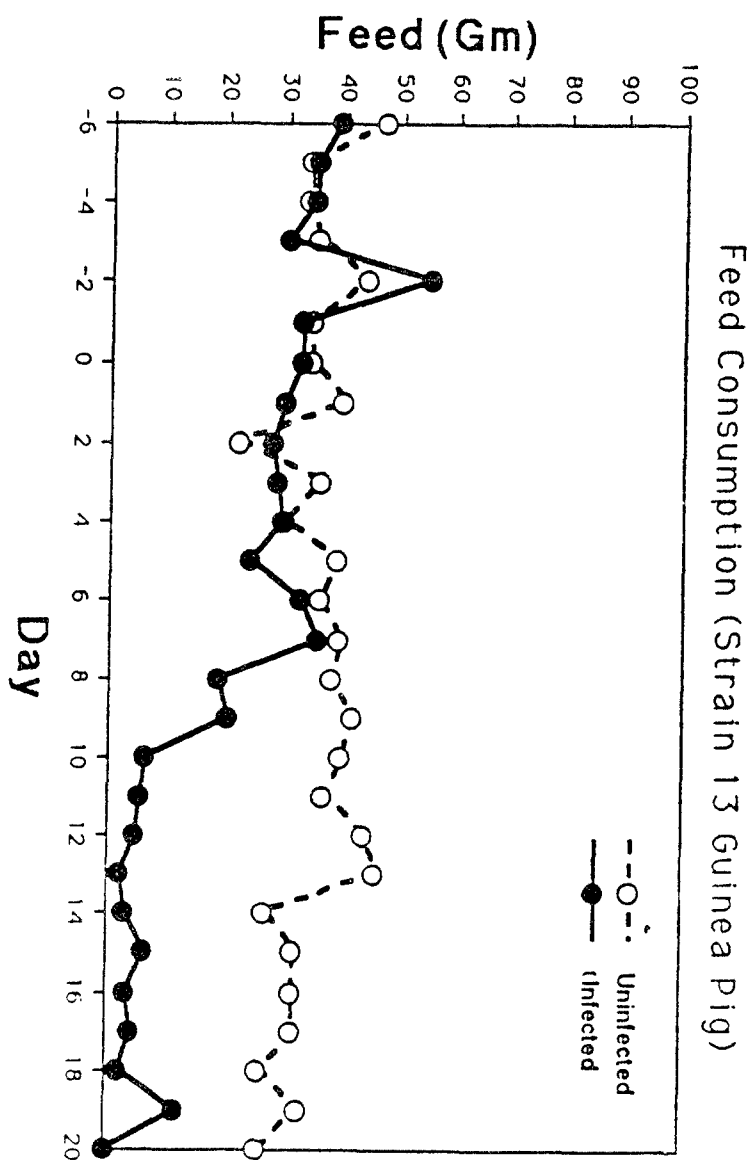
Figure 3. (A) Indirect immunofluorescent staining of Pichinde infected Vero cell culture using polyclonal anti-Pichinde primary antibody and FITC-labelled goat anti-hamster IgG secondary antibody (x400). (B) Pichinde-infected Vero cell cytospin preparation depicting typical granular, cytoplasmic localization of viral antigen (x1000).

Figure 4. Immunoperoxidase staining of Pichinde viral antigen in formalin-fixed paraffin embedded (FFPE) sections of Strain 13 guinea pig liver and spleen. (A) Positive Kupffer cell staining of liver at day 7 (x400). (B) Diffuse hepatocellular staining of liver at day 20 (x200). (C) Spleen at day 7 showing perifollicular antigen-positive macrophages (x200). (D) Spleen at day 18 with viral antigen disseminated throughout the red pulp (x200; rp=red pulp, wp=white pulp)

Figure 5. Pichinde viral antigen (immunoperoxidase) in FFPE sections of adrenal gland (early and late) and in sections of bronchiole and trachea of infected Strain 13 guinea pigs. (A) Positive focus in adrenal gland [zona fasciculata] at day 7 (x400). (B) Diffuse antigen staining of adrenal gland at day 18 (x200; zg=[zona glomerulosa], zf=[zona fasciculata]). (C) Antigen-positive staining of bronchiole epithelium at day 18 (x400). (D) Antigen-positive tracheal epithelium at day 20 (x400).

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Figure 6. Pichinde antigen (immunoperoxidase) in FFPE sections of (A) small intestinal villi (day 13; x400), (B) muscularis mucosa (day 13; x400) and, (C) vascular smooth muscle of urinary bladder (day 18; x400).

Figure 7. Sodium, chloride, BUN, and bicarbonate concentrations of Strain 13 guinea pigs inoculated with $2 \times 10\backslash3/$ PFU Pichinde virus. Points are arithmetic means, standard error < .05.
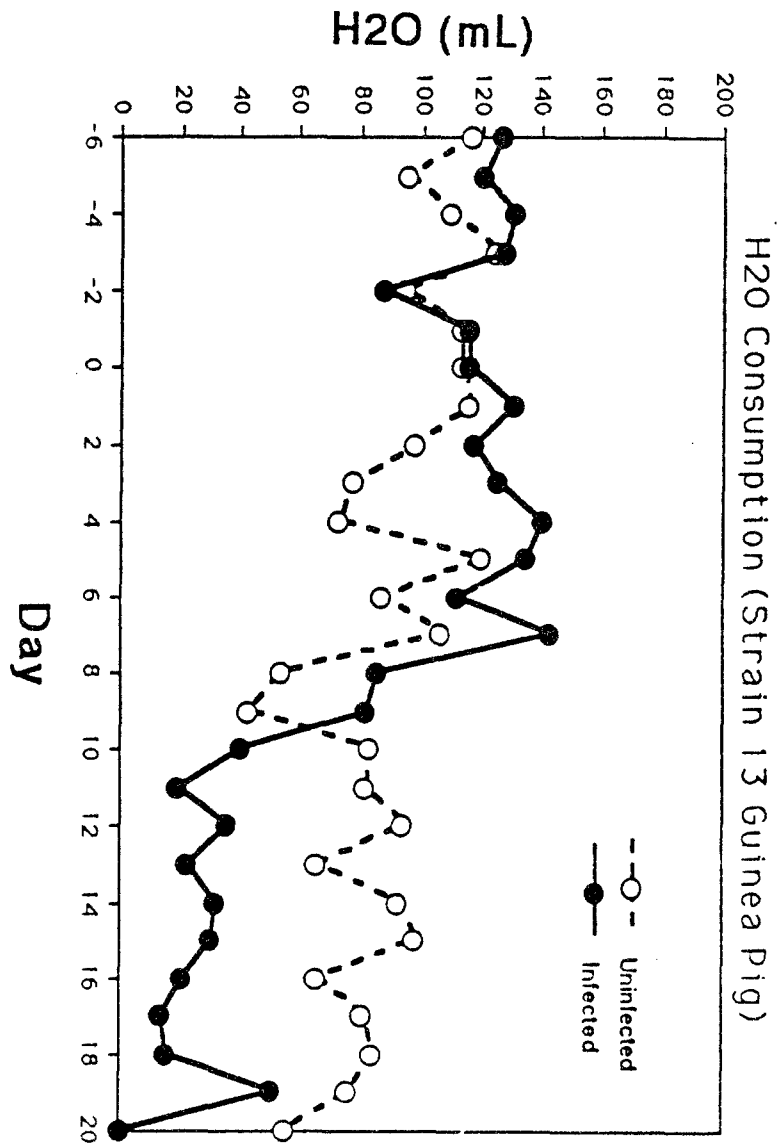
Figure 8. Total protein, albumin, triglyceride, and alkaline phosphatase concentrations of Strain 13 guinea pigs inoculated i.p. with $2 \times 10\backslash3/$ PFU Pichinde virus. Points are arithmetic means, standard error <.05.

FIGURE 1.A

Feed Consumption (Strain 13 Guinea Pig)

FIGURE 1. B

Figure 1. B — H2O Consumption (Strain 13 Guinea Pig), H2O (mL) vs. Day, comparing Uninfected and Infected groups.

FIGURE 1.C

% Weight Change (Strain 13 Guinea Pig)

FIGURE 2.

Log 10 PFU

Viral Titer (Strain 13 Guinea Pig)

Day

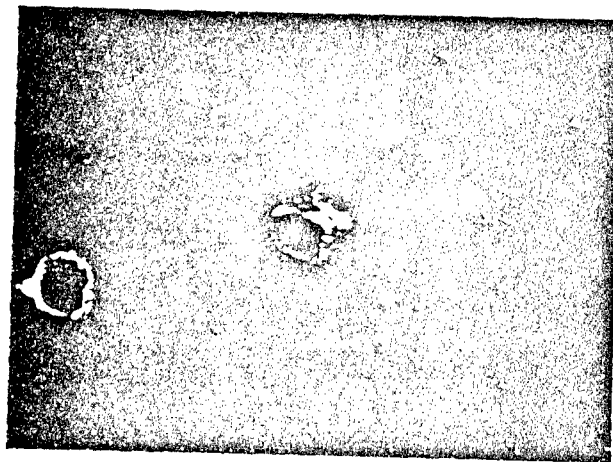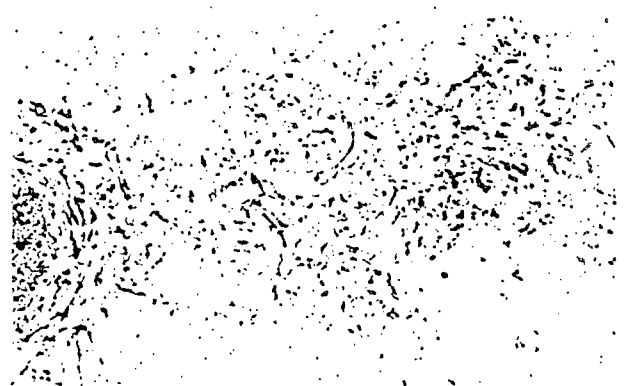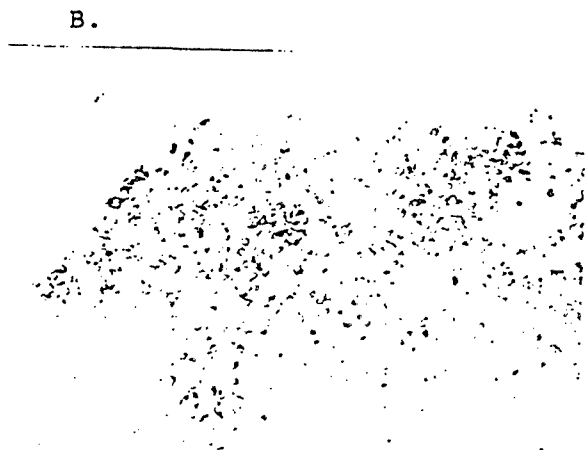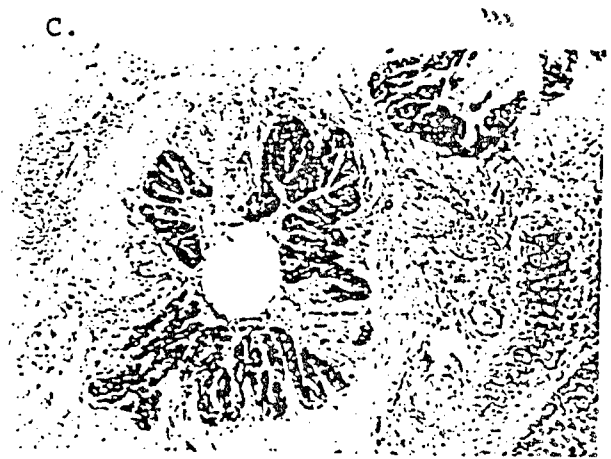Serum
Liver
Spleen
Brain
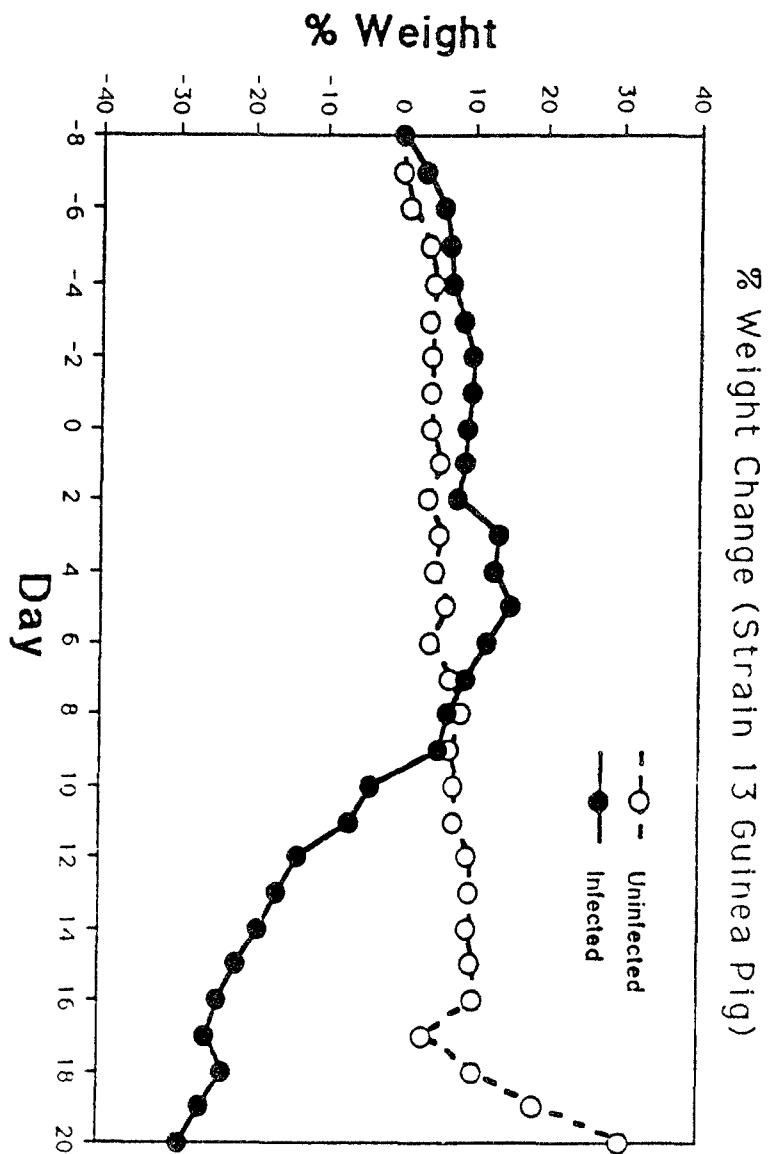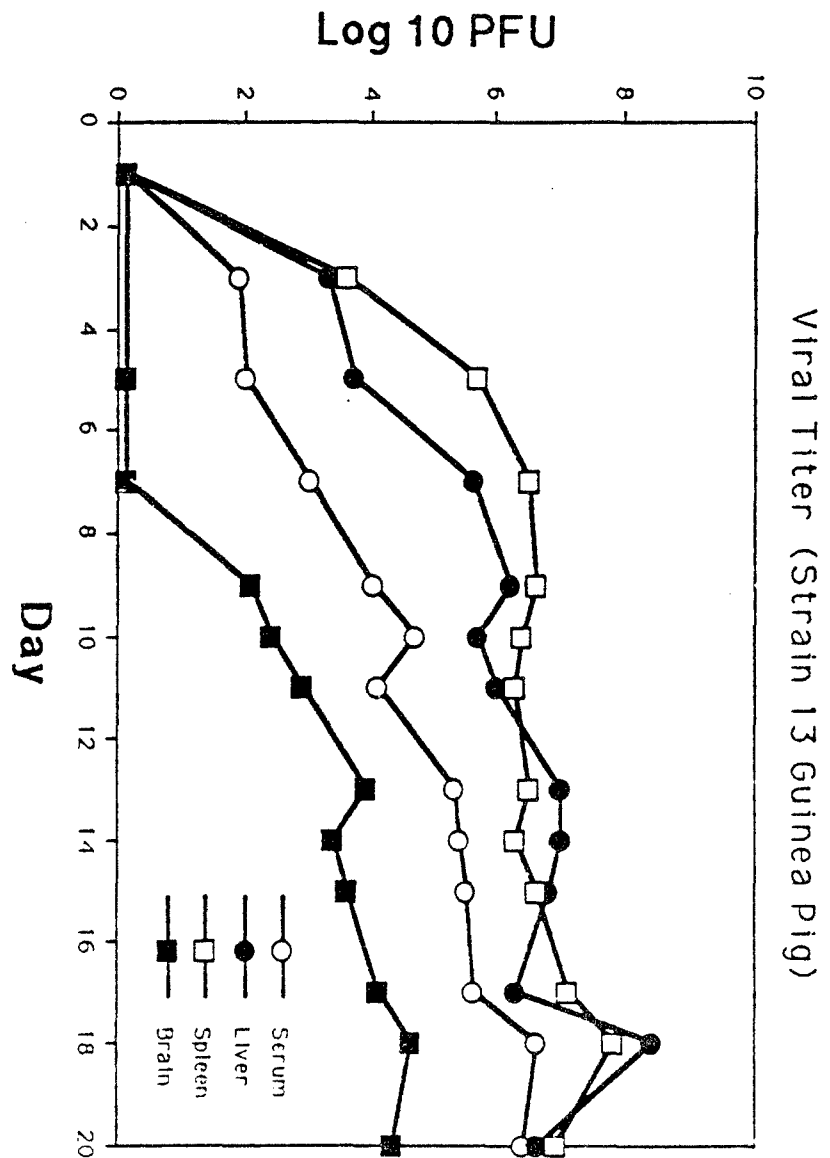
FIGURE 3.

A.
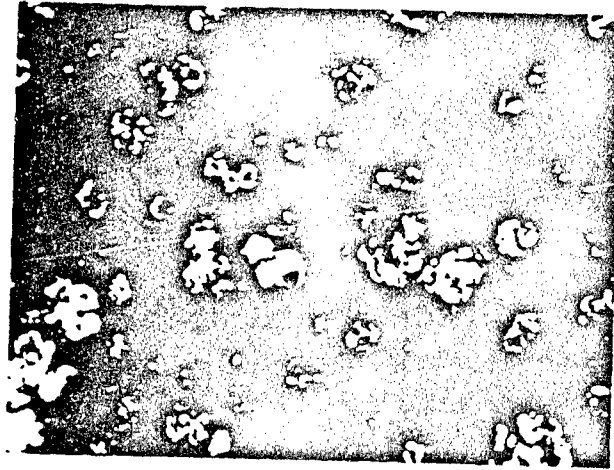
B.

FIGURE 4.

A.

C.

B.

D.

FIGURE 5.

FIGURE 6.

A.

B.

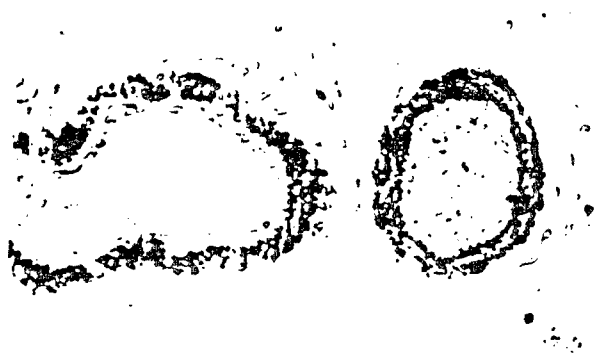

C.

FIGURE 7.

Bicarbonate (mEq/L)

Sodium (mEq/L)

BUN (mg/dL)

Chloride (mEq/L)

Infected
Uninfected

FIGURE 8.